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Potential of the microalgae *Nannochloropsis* and *Tetraselmis* for being used as innovative ingredients in baked goods

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Abstract

The potential use of the microalgae species *Tetraselmis* and *Nannochloropsis* was investigated for the production of functional breads and crackers. Optimum flour substitution levels were 2.5% for baked crackers and 1.0 or 2.0% for breads containing *Nannochloropsis* or *Tetraselmis*, respectively. No major differences were observed in the physicochemical properties of the end products besides an expected darker and greener colour. Microalgae incorporation led to increased phenolic content and *in vitro* antioxidant capacity in both matrices. For example, the total phenolic content of crackers increased from 24.6 ± 1.5 mg/100 g in the control to 32.4 ± 0.4 or 34.2 ± 1.0 mg/100 g in crackers containing *Tetraselmis* or *Nannochloropsis*, respectively. The amount of bioaccessible polyphenols after a simulated gastrointestinal digestion was also higher in microalgae-containing goods than in the controls. Sensory evaluation showed that microalgae-containing products were competitive with the controls with the added advantage of having an improved nutritional value and a "trendy" ingredient. Moreover, microalgae-containing products showed an increased emission of some volatile compounds such as p-cymene and (Z)-2-heptenal, which are responsible for fresh, citrus, terpenic, woody, and spicy or fatty, oily, and fruity odours, respectively.

Keywords: Functional foods, bread, crackers, *Nannochloropsis*, *Tetraselmis*.

1. Introduction

Humans are no strangers to the consumption of microalgae: already in the ninth century the Kanem Empire used *Arthrospira* as food in Africa (Oncel, Kose, Vardar, & Torzillo, 2015). Nowadays, microalgae are generally marketed as nutritional supplements and promoted as "superfoods" that can be utilised as ingredients in the manufacture of "trendy" foods. For example, baked goods formulated using microalgae such as Wrawp (Wrawp Foods, CA, USA) and Helga Algae Crackers (Evasis Edibles GmbH, Bendorf, Austria) are currently commercially available.

A large number of scientific publications evaluated the potential of *Spirulina* and *Chlorella* for being used as ingredients in the manufacture of milkshakes, vegetable soups, snacks, pasta, yogurts, and baked goods including bread and biscuits (Lafarga, 2019). This makes sense as both *Spirulina* and *Chlorella* are not only the most popular but also the most studied and cultivated microalgal strains (Garrido-Cardenas, Manzano-Agugliaro, Acien-Fernandez, & Molina-Grima, 2018). However, only a limited number of publications studied the effect of incorporating other species into this food group. For example, García-Segovia, Pagán-Moreno, Lara, and Martínez-Monzó (2017) reported that, although colour differences were observed when compared to the control, textural properties of the breads were not affected after incorporation of *Isochrysis galbana*, *Tetraselmis suecica*, *Scenedesmus almeriensis*, or *Nannochloropsis gaditana* at a concentration of 1.5% (w/w). Limited information is also available on the sensorial attributes of breads formulated using microalgae species different to *Spirulina* and *Chlorella*. Sensorial attributes of foods, especially flavour and aroma, are of key importance, as Western cultures do not seem to be willing to compromise taste for health. In addition, little is known on the effect of microalgae incorporation into other baked products different from bread. Some studies have been conducted on functional biscuits

enriched in: (i) eicosapentaenoic acid from *I. galbana* (Gouveia et al., 2008); (ii) fibre and protein from *S. platensis* (Singh, Singh, Jha, Rasane, & Gautam, 2015); and (iii) polyphenols and proteins from *S. platensis*, *C. vulgaris*, *T. suecica*, or *Phaeodactylum triconutum* (Batista et al., 2017). Biscuits or cookies are good delivery vehicles for health-promoting compounds because of their popularity and convenience. However, they contain a high sugar and/or fat (generally butter) content and it would be interesting to assess the effect of microalgae incorporation into other healthier products such as crackers.

Based on the current gap in knowledge, the aim of the current paper was to assess the potential of the species *Tetraselmis* and *Nannochloropsis*, which are currently underutilised in the food industry, for being used as novel ingredients for the production of functional breads and crackers. Studied quality parameters included volume, colour, texture, polyphenolic content, antioxidant activity, aroma volatile compounds, and sensorial attributes. In addition, the bioaccessibility of polyphenols after a simulated gastrointestinal digestion and the volatile profile of the products were also determined.

2. Materials and methods

2.1 Preparation of the microalgae-containing breads and crackers

Flour substitution levels evaluated in preliminary trials varied from 1 to 3% (w/w) for bread and from 1.25 to 3.75% (w/w) for crackers. Breads were produced following a straight dough baking procedure as described by Lafarga, Gallagher, Aluko, Auty, and Hayes (2016). Control wheat-only breads were labelled as BR-C. Breads containing *Tetraselmis* or *Nannochloropsis* at flour substitution levels of 2.0 or 1.0% (w/w) were labelled as BR-T and BR-N, respectively.

Crackers were produced following the methodology previously described by Lafarga et al. (2019a) with some modifications: in the current study, the doughs were sheeted to 2.0 mm instead of 2.5 mm and were cut in 40 mm circles instead of squares. Control crackers were labelled as CR-C and crackers containing *Tetraselmis* or *Nannochloropsis* biomass at a concentration of 2.5% (w/w) were labelled as CR-T or CR-N, respectively.

2.2 Physical analysis

Colour recordings (L^*, a^*, and b^* values) were taken using a Minolta CR-200 colorimeter (Minolta INC., Tokyo, Japan) and the D65 illuminant. Chroma (*Ch*) and difference from the control (ΔE) were calculated in triplicate as described by Lafarga et al. (2019b) and determined on day 1 post-baking.

The weight and dimensions of ten crackers were averaged for each formulation and replicate. Cracker dimensions were measured at day 1 post-baking using a digital Vernier calliper (JP Selecta, Barcelona, Spain) and the spread ratios, specific volume, and density were calculated for each cracker as described by Jan, Panesar, and Singh (2018). Bread loaf volume was calculated using AACC Method 10-05.01.

Moisture content was determined using AACC Method 44-15.02. The water activity (a_w) of all samples was measured using an AquaLab meter (Decagon Devices Inc., WA, USA). Three measurements were taken for each formulation and replicate. The pH of 1 g of ground sample, added to 10 g of distilled water, was determined in triplicate per formulation and replicate using a Basic 20 pH-meter (Crison Instruments S.A., Barcelona, Spain).

Texture characteristics were assessed using a TA.XT2 Texture Analyser (Stable Micro Systems Ltd., Surrey, England) connected to Exponent software v.5.0.6.0. Texture profile analysis of the breads was conducted as described by Lafarga et al. (2019c) and using a P/20 aluminium compression probe. Crackers hardness was determined using a knife edge with slotted insert probe (HDP/BS) as described by Lafarga et al. (2019a). Ten samples were taken for each formulation and replicate.

2.3 Total phenolic content

The total phenolic content (TPC) of the breads and crackers was determined by the Folin Ciocalteu method, following the protocol described by Lafarga, Villaró, Bobo, Simó, and Aguiló-Aguayo (2019d). Extraction time was 2 h at room temperature. TPC was determined in triplicate and results were expressed as mg of gallic acid equivalents per 100 g of dry weight (DW).

2.4 Antioxidant activity

The antioxidant capacity of the breads and crackers was determined using the same extract utilised for determination of TPC and using both the ferric reducing antioxidant power (FRAP) and the DPPH scavenging activity assays. The procedure followed was described previously by Lafarga et al. (2019d). Antioxidant capacity was determined in triplicate and results were expressed as mg of ascorbic acid equivalents per 100 g of DW.

2.5 *In vitro* gastrointestinal digestion

A simulated gastrointestinal digestion was performed in duplicate following the standardised static *in vitro* method previously described by Minekus et al. (2014). The method consists of three sequential states: (i) oral (37 ºC, pH 7.0, α-amylase, 2 min), (ii) gastric (37 ºC, pH 3.0, pepsin, 2 h) and (iii) intestinal (37 ºC, pH 7.0, pancreatin and fresh bile, 2 h). The pancreatin used contained enzymatic components including trypsin, amylase and lipase, ribonuclease, and protease. A blank was prepared using distilled water instead of sample. Determinations after the intestinal phase were performed in triplicate as described in previous sections.

2.6 Sensorial analysis

Sensory evaluation was undertaken by 30 semi-trained panellists (18 women, 12 men, age 18-50) recruited from IRTA Fruitcentre (Lleida, Spain) at day 1 post-baking. Sensory evaluation was conducted following the methodology described by Lafarga et al. (2019a). Each panellist assessed all the samples and was asked to indicate her or his opinion on the firmness, flavour, overall visual appearance, and overall acceptability of the products using a 9-point hedonic scale (from 1: extremely dislike to 9: extremely like). The acceptability index (AI) was calculated as described by Lucas, Morais, Santos, and Costa (2018). Finally, purchase intention (PI) was assessed using a 5-point hedonic scale which ranged from 1: "certainly would not buy" to 5: "certainly would buy".

2.7 Volatile compounds

Extraction and determination of the volatile compounds emitted by the breads and crackers was performed using HS-SPME-GC/MS following the conditions previously described by Pico, Antolín, Román, Gómez, and Bernal (2018) with some modifications. Briefly, an amount of 1 g (±0.005 g) of ground sample was weighed into 20 mL vials and

mixed with 10 mL of 20% (w/v) sodium chloride at pH 3.0. The vials were immersed in a water bath at 60 ºC and the SPME fibre (65 mm PDMS/DVB; Supelco Co., PA, USA) was exposed to the headspace for 60 min.

After extraction, the fibre was injected for thermal desorption into the injector port for 10 min. The GC-MS analyses were performed using a 6890N gas chromatograph-mass spectrometer equipped with a HP-FFAP (50 m × 0.2 mm; 0.33 µm) column, both purchased from Agilent Technologies Inc. (CA, USA). Temperature conditions are described in the above cited publication. In this study, injector and detector temperatures were 240 ºC. Mass spectra were obtained by electron impact ionisation at 70 eV and the scan mode was used to detect all the compounds in the range m/z 20-350. The preliminary identification of volatile compounds was verified by comparison of the mass spectral data obtained with those in NIST62 mass spectral database.

2.8 Statistical analysis

Results are expressed as mean ± standard deviation (S.D.). Differences between samples were analysed using analysis of variance (ANOVA) with JMP 13 (SAS Institute Inc., Cary, USA). Where significant differences were present, a Tukey pairwise comparison of the means was conducted to identify where the sample differences occurred ($p<0.05$).

3. Results and discussion

3.1 Preliminary baking trials

Incorporation of *Tetraselmis* and *Nannochloropsis* biomass into bread and crackers significantly affected colour parameters ($p<0.05$): ΔE was higher than 3 for all the formulated breads and crackers, suggesting that colour differences with the control were visible to the human eye. Higher microalgae content led in bread formulations to lower L^* values for both crust and crumb ($p<0.05$; Figure 1): a negative correlation was observed between microalgal biomass concentration and L^* values in crust (0.905; 0.05) and crumb (0.817; 0.05). Crackers with higher microalgal biomass concentration showed lower L^* values, suggesting a darker colour ($p<0.05$; Figure 2). Similar results were reported previously (Figueira, Crizel, Silva, & Salas-Mellado, 2011; Menezes, Coelho, Meza, Salas-Mellado, & Souza, 2015). Although a^* values of the microalgae-containing breads were lower and b^* values were higher, when compared to the control, incorporation of higher concentrations of microalgae did not cause further differences in a^* and b^* values. These results may seem unexpected but this same effect was reported in baked products containing *S. platensis* (Batista et al., 2017), *C. vulgaris* (Gouveia, Batista, Miranda, Empis, & Raymundo, 2007), and *I. galbana* (Gouveia et al., 2008) and has been attributed to pigment degradation during the baking process and/or to a pigment saturation effect above a certain microalgae concentration.

Before discussing the sensorial acceptance of the breads and crackers it is important to highlight that panellists were first asked if they would be willing to buy baked products enriched in microalgae and only those who answered "yes" conducted the sensorial analysis. Moreover, results on sensorial analysis must be taken with caution, especially those on overall acceptance and PI, as the ideal would have been to assess these parameters using ~100 consumers. For a product to be accepted in terms of sensorial

characteristics, it is necessary to obtain an AI greater than 70% (Lucas et al., 2018). Although microalgae incorporation into the bread led to lower overall acceptability scores (p<0.05), formulated breads showed AI values ranging between 71.7 and 80.8%. These values are in line with those reported for other foods containing microalgae (Lafarga et al., 2019b; Lucas et al., 2018). Maximum AI was obtained for breads containing *Tetraselmis* at a concentration of 2.0% and *Nannochloropsis* at a concentration of 1.0%. These breads showed relatively high PI values: approximately 55% of the panellists said they "probably would buy" them. Approximately the same amount of panellists suggested that they "probably would buy" the control breads, although these showed a higher percentage of panellists who "certainly would buy" them. Overall acceptability of crackers was not affected after incorporation of microalgae into the recipe. All of the microalgae-containing crackers showed AI values over 70%. Crackers containing *Tetraselmis* and *Nannochloropsis* at a flour substitution level of 2.5% (w/w) showed AI values of 85.9 and 79.8%, respectively. Approximately 82 and 91% of the panellists scored these two crackers within the range 7-9 (between "like moderately" and "like extremely") and their PI ranged between 4 and 5 (between "would probably buy" and "certainly would buy"). Microalgae concentration higher than 2.5% resulted in decreased AI and PI values (p<0.05).

Based on these results, breads containing *Tetraselmis* at a concentration of 2.0% (w/w) or *Nannochloropsis* at a flour substitution level of 1.0% (w/w) were selected for further analysis. These were labelled as BR-T and BR-N, respectively. Moreover, crackers containing *Tetraselmis* or *Nannochloropsis* at a flour substitution level of 2.5% (w/w), which were labelled as CR-T and CR-N were also selected for further analysis.

3.2 Physicochemical properties

3.2.1 Colour and volume

No differences were observed between the colour attributes of the breads at day 3 post-baking (data not shown) when compared to those measured at day 1 (Figure 1) - except for a decrease in crust L^* values ($p<0.05$), probably caused by a loss of moisture during storage. Moreover, no colour differences were detected during storage of crackers for 10 days. Colour values during storage suggest a stable product in terms of visual appearance. In the current study, the specific volume of BR-T and BR-N was lower than that of BR-C ($p<0.05$; Table 1). Results can be attributed to a dilution of starch and gluten after substituting flour with microalgae and a decrease in the amount of fully hydrated starch granules caused by the added powder competing for water with starch. The lower loaf volume obtained after incorporation of microalgae into the recipe led to higher density in BR-N when compared to BR-C ($p<0.05$). In addition, microalgae-incorporation into the crackers formulation did not affect volume and density, suggesting that higher microalgae concentrations can be incorporated into crackers without negatively affecting the visual appearance of the products (when compared to bread). A high spread ratio, which is a quality measure, is desirable in baked products (Mudgil, Barak, & Khatkar, 2017). The spread ratio of the crackers was not affected after the incorporation of microalgae into the crackers' recipe. Previous studies suggested an increase of the spread ratio of crackers enriched in powdered broccoli co-products and reported a positive correlation between spread ratio and broccoli content (Lafarga et al., 2019a). Higher microalgae concentration could probably lead to higher spread ratios, although this would need to be assessed in further studies.

3.2.2 Moisture and water activity

Moisture content and a_w values of the breads was comparable to that measured in the crumb of commercially available bagels or breads (Schmidt & Fontana, 2008). Incorporation of microalgae into the bread formulations led to lower moisture content

(p<0.05). The moisture content of BR-T was lower than that of BR-N (p<0.05). A decrease in moisture was observed at day 3 post-baking because of bread staling (p<0.05). Water loss during storage was calculated as 18.5, 9.0, and 5.3% for BR-C, BR-T, and BR-N, respectively. Microalgae incorporation into the crackers also led to reduced humidity at day 1 post-baking (p<0.05). However, no significant differences were observed between the moisture content at days 1, 5, and 10 post-baking, suggesting stable products.

Substituting wheat flour with microalgal biomass did not affect the pH and the a_w of the breads at day 1 post-baking (Table 1). A decrease in a_w was observed in bread samples during storage (p<0.05). The observed decrease was bigger in BR-C when compared to BR-T and BR-N, probably caused by a higher moisture loss during storage. Similar a_w values were also observed in breads enriched in bioactive ingredients (Lafarga et al., 2016). The a_w of CR-T and CT-N was lower than that CR-C, caused by the above mentioned lower moisture content. Storage for 10 days did not affect pH and a_w values for any of the cracker samples, suggesting once again stable products.

3.2.3 Textural properties

Figure 3 shows the textural properties of BR-C, BR-T, and BR-N at days 1 and 3 post-baking. A higher bread density has often been correlated with increased hardness. However, in the current study, no differences were observed in hardness, which is the peak force that occurred during the compression of the bread slices. Similar results were observed after incorporation of freeze-dried broccoli co-products into bread at a concentration of 2% (Lafarga et al., 2019c). The observed increase in hardness at day 3, when compared to the values obtained at day 1, can be attributed to bread staling and moisture loss. Moreover, no differences in springiness, cohesiveness, gumminess, chewiness, and resilience were observed between the microalgae-containing breads BR-

T or BR-N and BR-C, suggesting a comparable mouth-feel and a similar retention of the textural properties after compression at both days 1 and 3 post-baking. Results were in line with those reported by García-Segovia et al. (2017).

Hardness of the control and microalgae-containing crackers, which is the force required to break or snap the cracker, is shown in Figure 3. Lower moisture content is correlated with increased hardness in crackers (Millar et al., 2017). However, no significant differences between the hardness of CR-T, CR-N, and CR-C. As shown in Figure 3, no differences in hardness were observed during storage. Similar results were observed previously in other food matrices where microalgae incorporation did not affect functional properties of the end products (De Marco, Steffolani, Martínez, & León, 2014; García-Segovia et al., 2017).

3.3 Total phenolic content and antioxidant capacity

Currently, algae-derived polyphenols are one of the top trends in functional foods for the prevention of cardiovascular diseases and diabetes (Murray, Dordevic, Ryan, & Bonham, 2018). Microalgae incorporation led to increased TPC in both studied food matrices (p<0.05) and, as expected, to an increased antioxidant capacity (Figure 4). Results are not surprising as several studies reported the high antioxidant activity of microalgal biomass, which has been attributed to their high phenolic and carotenoid content (Goiris et al., 2012). A positive correlation was observed between TPC and antioxidant capacity of crackers at when assessed using the FRAP (0.884 at day 1 and 0.986 at day 5; 0.05) and DPPH (0.852 at day 1 and 0.991 at day 5; 0.05) methods. Previous studies also reported an increased content of polyphenols and a higher antioxidant capacity after incorporation of microalgae in, for example, pasta (De Marco et al., 2014) or broccoli soup (Lafarga et al., 2019b).

Results shown in Figure 4 demonstrate that the amount of polyphenols in the enzymatic digestive extracts obtained after a simulated gastrointestinal digestion is higher than that expected based on extractions made using methanol ($p<0.05$). This was probably caused by a higher liberation of polyphenols because of the action of digestive enzymes. The longer extraction time can also partially explain these findings. Not only the phenolic content but also the antioxidant capacity of the enzymatic digestive extracts was higher than that of the methanolic extracts ($p<0.05$). The observed increase in antioxidant capacity can be attributed to the higher phenolic content but also to the generation of bioactive peptides with antioxidant capacity, as previous studies demonstrated that microalgae are good sources of bioactive peptides (Ko et al., 2018; Wu, Xu, Sun, Yu, & Zhou, 2015). Food processing is a crucial step to improve bioaccessibility and produce products with beneficial nutritional properties (Barba et al., 2017). Cavonius, Albers, and Undeland (2016) suggested that cell disruption, and to a lesser extent, strong pH variations were needed to increase bioaccessibility of lipids while a pre-freezing step was required to improve accessibility of proteins derived from *Nannochloropsis oculata*. The bioaccessibility and bioavailability of other antioxidant compounds found in microalgae such as carotenoids have been shown to strongly depend on, for example, the food matrix and processing conditions (Kopec and Failla, 2018).

3.4 Sensorial attributes and volatile profile

Incorporation of microalgae into the bread formulation, at the concentrations studied herein, did not affect the visual appearance scores of the breads. Lafarga et al. (2019c) recently reported high visual acceptability scores of a green bread formulated using broccoli leaves. No differences were observed in the texture and flavour scores of BR-C and BR-T or BR-N, although some panellists (3 out of 30) described an unpleasant "fishy" taste. Incorporation of microalgae into the bread formulation led to lower aroma scores

312 (p<0.05). The aroma score of BR-T was lower than that of BR-N (p<0.05), probably
313 caused by a higher microalgae content. Incorporation of microalgal biomass into the
314 cracker formulation led to a decreased visual appearance when compared to CR-C
315 (p<0.05; Figure 5). Although green crackers containing *Chlorella* biomass, at a
316 concentration of 5%, are currently being commercialised under the brand Helga Algen
317 Cracker (Evasis Edibles, Austria), the number of green-coloured baked goods currently
318 being commercialised in Europe is still limited. European consumers are not yet used to
319 coloured baked products and this could be the cause of the observed lower visual
320 appearance scores. Moreover, no differences were detected between the aroma scores of
321 CR-T, CR-N, and the control CR-C. In turn, flavour and overall acceptance scores were
322 higher in microalgae-containing crackers when compared to the control (p<0.05) – don't
323 forget that only panellists that would be willing to buy microalgae-enriched products
324 carried out the sensorial analysis. As mentioned previously, these results are preliminary
325 and a sensorial analysis with a larger number of consumers would better describe the
326 sensorial attributes and the acceptance of these products.
327 Volatile compounds identified in the breads and crackers are listed in Table 2. A total of
328 42 compounds including alcohols (5), aldehydes (12), ketones (4), esters (11), sulfur
329 compounds (2), acids (1), terpenes (4) and hydrocarbons (3) were detected in the samples.
330 The most abundant compounds in crackers were (listed in decreasing order) undecanone,
331 hexanoic acid, dipropyl disulfide, nonanal, phenylacetaldehyde, 3-methyl-1-butanal, and
332 2-methyl-1-butanal. Regarding aldehydes, the most important odorant was nonanal,
333 which is formed from β-cleavage of the 10-OOH hydroperoxide and imparts green and
334 fatty notes to flavour (Parker, 2015). Among the Strecker aldehydes of the amino acid
335 methionine, phenylacetaldehyde and 2- and 3-methylbutanal were the most prominent in
336 the aroma profile of crackers, which were also detected in previous HS-SPME studies on

wheat bread (Raffo, Carcea, Castagna, & Magrì, 2015). Most relevant alcohols were 3-methyl-1-butanol, 1-octen-3-ol, and 2-ethyl-1-hexanol. 3-Methyl-1-butanol is produced during dough fermentation (Mildner-Szkudlarz, Zawirska-Wojtasiak, Szwengiel, & Pacyński, 2011). In the group of ketones, besides identifying aroma compounds such as 1-octen-3-one, 6-methyl-5-hepten-2-one, and 2-nonanone, we would like to highlight the identification of 2-undecanone, which was the most abundant ketone in this study and was not reported in previous HS-SPME analyses on bread (Pacyński, Wojtasiak, & Mildner-Szkudlarz, 2015). The groups of esters, acids, sulphides, terpenes, and hydrocarbons completed the list of identified compounds. The aroma profile of bread and crackers was similar. However, in bread the most significant aldehyde was (Z)-2-heptenal, which is a product from the degradation of the linoleic acid (Parker, 2015).

4. Conclusions

Overall, *Tetraselmis* and *Nannochloropsis* biomass show potential for being used as novel functional ingredients in bread and crackers. Results demonstrated that not only the *in vitro* phenolic content or antioxidant capacity of the products was improved after microalgae-incorporation but also the amount of bioaccessible polyphenols and the antioxidant capacity of the enzymatic digestive extracts, suggesting also healthier products. Their utilisation would also allow food processors to differentiate by using a "trendy" ingredient. Sensory evaluation showed that microalgae-containing breads and crackers, enriched at the concentrations studied in the current study, were competitive with the control breads and crackers with the added advantage of having an improved nutritional value.

Acknowledgements

This work was supported by the CERCA Programme of *Generalitat de Catalunya*. T. Lafarga (FJCI-2016-29541) and I. Aguiló-Aguayo (RYC-2016-19949) thank the Spanish Ministry of Economy, Industry, and Competitiveness. All authors thank Fundación Cajamar (Almería, Spain) for kindly providing with the microalgal biomass.

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